

Secondary Lipid Oxidation Products as Modulators of Calpain-2 Functionality *In Vitro*

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ABSTRACT: The objective was to understand the impacts of secondary lipid oxidation products on calpain-2 activity and autolysis and, subsequently, to determine the quantity and localization of modification sites. 2-Hexenal and 4-hydroxynonenal incubation significantly decreased calpain-2 activity and slowed the progression of autolysis, while malondialdehyde had minimal impact on calpain-2 activity and autolysis. Specific modification sites were determined with LC-MS/MS, including distinct malondialdehyde modification sites on the calpain-2 catalytic and regulatory subunits. 2-Hexenal modification sites were observed on the calpain-2 catalytic subunit. Intact protein mass analysis with MALDI-MS revealed that a significant number of modifications on the calpain-2 catalytic and regulatory subunits are likely to exist. These observations confirm that specific lipid oxidation products modify calpain-2 and may affect the calpain-2 functionality. The results of these novel experiments have implications for healthy tissue metabolism, skeletal muscle growth, and post-mortem meat tenderness development.

KEYWORDS: calpain-2, lipid oxidation products, mass spectrometry, post-translational modifications

INTRODUCTION

In biological systems, the degradation of lipids or lipid peroxidation can yield a wide variety of secondary oxidation products, including alkanals, alkenals, 4-hydroxy-alkenals, 4-keto-alkenals, and alkanedials, which can in turn lipoxidate proteins.¹ Compounds such as malondialdehyde, acrolein, 4-hydroxyhexanal, and 4-hydroxy-2-nonenal have been most extensively studied as protein lipoxidation modifications,² and additional compounds, such as 2-hexenal,^{3–6} hexanal,⁷ 4-oxo-2-nonenal,⁸ and others,⁹ have been less studied. Purified or isolated proteins, including lactate dehydrogenase,¹⁰ pyruvate kinase,¹¹ cytochrome c,^{12,13} cytochrome c oxidase,¹⁴ β -lactoglobulin B,^{15,16} hemoglobin,^{15,17,18} insulin,^{3,19} myoglobin,^{20–29} apomyoglobin,^{8,30,31} and calpain-1,³² are modified by lipid oxidation products in *in vitro* systems. Functional protein analysis combined with the mass spectrometry approach has demonstrated almost exclusive negative impacts of lipid oxidation on protein functionality and has provided insights into mechanistic modifications, which partly explain the differences in protein functionality. While not all proteins may be equally impacted, modifications by lipid oxidation products have been recognized to negatively impact the function of some proteins *in vitro*.

Calpains are a family of intracellular calcium-dependent cysteine proteases.³³ Calpain-1 and calpain-2 are heterodimers containing distinct 80 kDa subunits and identical 28 kDa subunits, and their activation requires both the presence of calcium and a reducing environment.^{33,34} Calpains have been consistently identified as an important protease system involved in a myriad of cellular processes, generally through the degradation of substrates or enzymes important for these processes, including cytoskeletal remodeling, signal trans-

duction, gene expression, myoblast fusion, and apoptotic pathways.^{33,35} Calpain-2 is specifically crucial for successful myoblast fusion *in vitro*.³⁶ Calpain-2 knockout leads to embryonic lethality in mice during the preimplantation stage, while calpain-1 cannot compensate for this function of calpain-2.^{37,38} Strategies to use isoform-specific knockouts and knockdowns *in vitro* and *in vivo* have been conducted to explore the distinct roles of calpain-1 and calpain-2.^{39–41} These studies have revealed that calpain-2 plays crucial roles in embryonic and myogenic development and that changes to calpain-2 can result in cellular dysfunction or cell death. Previous studies demonstrated the negative impacts of post-translational modifications by lipid oxidation products on various proteins. Thus, the modification of calpain-2 by lipids and other oxidation products could have negative implications in cellular environments.

The calpain family of proteases has also been implicated in the development of meat tenderness development. Calpain-1 activity and autolysis are closely related to improvements in post-mortem meat tenderness,⁴² and the degradation of many proteins associated with meat tenderness development are similarly degraded under *in vitro* conditions with calpain-1.⁴³ Calpain-1 has been demonstrated to be a primary driver of meat tenderness development, especially earlier post-mortem, due to a lower calcium requirement.⁴⁴ Desmin is a substrate of

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calpain-2,⁴⁵ and oxidation by hydrogen peroxide and a decrease from neutral pH separately and collectively impact calpain-2 activity⁴⁶ and the rate of desmin degradation *in vitro*.⁴⁵ Protein carbonylation occurs during post-mortem aging of skeletal muscles,⁴⁷ and these oxidative modifications alter the functionality of some proteins, including calpain-1.^{48,49} The impact of calpain-2 on post-mortem proteolysis and meat tenderness development is still unclear, as changes in pH, ionic strength, temperature, oxidative environment, and other factors post-mortem may limit its proteolytic activity. However, the improvement in tenderness with extended post-mortem aging suggests that calpain-2 also plays a role in protein proteolysis as calcium concentrations increase in the sarcoplasm^{50,51} and calpain-2 autolysis occurs.^{50,52–54} We hypothesize that the accompanying generation of lipid oxidation products during post-mortem aging could further limit the proteolytic activation of calpain-2.

Therefore, the objective of this study was to determine the impact of specific lipid oxidation products, including malondialdehyde, 2-hexenal, and 4-hydroxynonenal, on calpain-2 activity and autolysis and to identify specific modifications of calpain-2 by these lipid oxidation products using mass spectrometry approaches. Based on previous studies, it was hypothesized that calpain-2 activity and progression of autolysis would decrease and slow down, respectively, and that specific lipoxidation modifications linked to alterations in calpain-2 function would be identified.

MATERIALS AND METHODS

Purification of Calpain-2. Calpain-2 was purified from porcine *semimembranosus* skeletal muscle collected approximately 45 min post-mortem using successive column chromatography as described.⁴⁵ One unit of calpain-2 activity was defined as the amount necessary to generate an increase in the absorbance of trichloroacetic acid-soluble peptides at 278 nm by 1 U after 1 h of incubation at 25 °C with the activity assay buffer (3.5 mg/mL casein, 40 mM Tris-HCl [pH 7.4], 5 mM CaCl₂, 0.1% β -mercaptoethanol [MCE], at 25 °C).⁵⁵ The purified calpain-2 used in the experiment had a specific activity of 120–125 units/mg protein, which is similar yet slightly less pure compared to previous studies with purified calpain-2.^{45,46}

Influence of Lipid Oxidation Products on Calpain-2 Activity. The stock solution (10 mM) of malondialdehyde tetrabutylammonium salt (malondialdehyde; 63287-1G-F, Sigma-Aldrich) was prepared in ddH₂O, while the stock solutions (10 mM) of 2-hexenal (H0345, TCI America) and 4-hydroxynonenal (32100, Cayman Chemical) were prepared in 100% ethanol. Pure calpain-2 was dialyzed against a TEM buffer (40 mM Tris-HCl [pH 7.4], 1 mM ethylenediaminetetraacetic acid [EDTA], and 0.1% MCE) for 3 h at 4 °C using Slide-A-Lyzer Mini dialysis units with a molecular weight cutoff (MWCO) of 7000 Da (Pierce, Rockford, IL). Calpain-2 (5 μ g) was incubated with 100, 500, or 1000 μ M of malondialdehyde, 2-hexenal, or 4-hydroxynonenal, or equal volumes of TEM (controls) buffer, for 24 h at 4 °C. The concentrations utilized throughout this study were based on previous *in vitro* studies of purified proteins^{13,27,32} and were exploratory and titrated across three different concentrations. These concentrations are likely greater than those achieved *in vivo* in living and post-mortem skeletal muscles, but further characterization of these lipid oxidation product concentrations *in vivo* is warranted. After incubation, calpain-2 activity was determined in triplicate using a caseinolytic assay⁵⁵ and replicated six ($n = 6$) times on separate days. The activity of calpain-2 after incubation with less than 20% ethanol for 24 h at 4 °C was 95.7% compared to that of TEM controls.

Influence of Lipid Oxidation Products on Calpain-2 Autolysis. Pure calpain-2 was dialyzed against TEM using Slide-A-Lyzer Mini dialysis (MWCO 7000 Da; Pierce) units for 3 h at 4 °C. The dialyzed calpain-2 (30 μ g) was incubated with 1000 μ M of

malondialdehyde, 2-hexenal, or 4-hydroxynonenal, or equal volumes of the solvent of oxidant stock solutions (controls), for 24 h at 4 °C. After incubation, an aliquot of calpain-2 was removed, which served as the no-calcium control, and immediately placed in the protein-denaturing buffer (3 mM EDTA, 3% [wt/vol] sodium dodecyl sulfate [SDS], 30% [vol/vol] glycerol, 0.001% [wt/vol] pyronin Y, and 30 mM Tris-HCl [pH 8.0]) and 0.1 vol of MCE.⁵⁶ Subsequently, each preparation was then adjusted to a final concentration of 2 mM CaCl₂ and incubated on ice. Aliquots were removed at 5, 15, and 60 min after the addition of CaCl₂ and immediately quenched in a protein-denaturing buffer. All samples were heated on a dry heat block for 15 min at 50 °C. Each sample (1.5 μ g) was resolved on 15% polyacrylamide separating gels as previously described.⁴⁴ Following electrophoresis, gels were visualized using the FASTsilver Gel Staining Kit (Sigma-Aldrich) according to the manufacturer's instructions and imaged using a ChemiImager 5500 Imager (Alpha Innotech Corp). Replicate analyses ($n = 2$) for the control and each oxidant were conducted on separate days.

Purified Calpain-2 for LC-MS/MS and MALDI-MS. Pure calpain-2 was dialyzed against either TEM (reducing) or TE (nonreducing; 40 mM Tris-HCl [pH 7.4] and 1 mM EDTA) for 2 h at 4 °C using Spectra/Por 4 dialysis tubing (MWCO 12–14 kDa; Spectrum, Rancho Dominguez, CA). Dialyzed calpain-2 (125 μ g) was incubated separately with 1000 μ M malondialdehyde, 2-hexenal, 4-hydroxynonenal, or 20% ethanol (control) for 24 h at 4 °C. After incubation, each calpain-2 sample was dialyzed separately using Slide-A-Lyzer Mini dialysis units against at least 100 vol of TE for 30 min at 4 °C, and samples were stored at –80 °C until analysis. Two replicates of the control and each oxidant by reducing and nonreducing environments were conducted, resulting in 16 samples for LC-MS/MS and MALDI-MS analyses.

LC-MS/MS Sample Preparation and Analysis. Samples were processed using the EasyPep Mini MS Sample Prep Kit (Thermo Fisher Scientific) following the manufacturer's instructions with one modification. Briefly, samples were supplemented with 1 mM CaCl₂ to minimize the impact of EDTA on the digest. 44 μ g of protein was aliquoted from each sample and the volume was increased to 100 μ L with lysis buffer. Reduction and alkylation solutions (EasyPep Mini MS Sample Prep Kit; Thermo Fisher Scientific) were sequentially added, followed by incubation at 95 °C for 10 min. After the mixture was cooled to room temperature, 10 μ g of trypsin/LysC mixture was added, and samples were digested with shaking at 37 °C for 3 h. The enzymes were then deactivated with the digestion stop solution, and contaminants were removed using mixed-mode peptide cleanup columns. The peptide eluate was dried in a vacuum evaporator and resuspended in 5% acetonitrile/0.1% formic acid. Once resolubilized, the absorbance was measured at 205 nm on a NanoDrop (Thermo Scientific), and the total peptide concentration was subsequently calculated using an extinction coefficient of 31.⁵⁷

Reverse-phase chromatography was performed with 0.1% formic acid as buffer A and 80% acetonitrile with 0.1% formic acid as buffer B. 800 ng of peptides was purified and concentrated using an online PepMap Neo trap cartridge (0.5 cm $\times$ 300 μ m inner diameter) packed with 5 μ m diameter, 100 Å pore size, and ultrapure silica C18 particles (Thermo Scientific). A Vanquish Neo UHPLC system (Thermo Scientific) with a PepMap nanospray column (15 cm $\times$ 75 μ m inner diameter, 40 °C) packed with 2 μ m diameter, 100 Å pore size, and fused silica C18 particles was used for subsequent chromatographic separations. The peptide mixture was separated at 300 nL/min with a 30 min gradient: 1–6% B over 30 s, followed by 6–45% B over 25 min, and finally, a 99% B wash for 4.5 min. Peptides were eluted directly into an Orbitrap Eclipse (Thermo Scientific) mass spectrometer operated in positive ionization mode equipped with a Nanospray Flex ion source (Thermo Scientific). Spectra were collected over 375–2000 m/z . Ions with a charge state of +2 or higher were accepted for MS/MS using a dynamic exclusion limit of 1 MS/MS spectra of a given m/z value with an exclusion duration of 7 s. The instrument was operated in FT mode for MS detection (profile; resolution of 240,000) and ion trap mode for MS/MS detection with the normalized high collision energy set to 30% and data centroided.

Tandem mass spectra were mass shift calibrated, and peaks were detected using the Spectrum Files RC and Minora Feature Detector nodes in Proteome Discoverer (ver. 3.0, Thermo Scientific). Searches were conducted against the UniProt *Sus scrofa* reference proteome (UP000008227, downloaded January 24, 2022). Spectra searches utilized an initial Sequest HT⁵⁸ node with a precursor mass tolerance of 10 PPM and a fragment mass tolerance of 0.60 Da, assuming the digestion enzyme trypsin and a maximum of two missed cleavages. The dynamic modifications were methionine oxidation (+15.995 Da), carbamidomethylation of cysteine residues (+57.021 Da), and acetylation of the protein N terminus (+42.011 Da). Two additional Sequest HT nodes were included to separately search for dynamic MDA, HXL, and HNE modifications of cysteine, histidine, lysine, glutamine, arginine, and asparagine residues, including various product masses as described previously.³² Peptide spectrum matches (PSM) were validated using the Percolator node⁵⁹ with a false discovery rate (FDR) of $\leq 1\%$. Protein identification was defined by at least one identified peptide, and proteins were grouped to satisfy the principles of parsimony. Spectra and fragmentation tables were evaluated to confirm adductions of secondary lipid oxidation products through the manual assessment of identified fragment ions with a precursor mass error of less than 5 PPM. Representative spectra and fragmentation tables of modified peptides are included in the [Supporting Information](#). The heteromeric complex of calpain-2 catalytic (UniProt ID: A0A5G2QB32) and regulatory (UniProt ID: P04574) subunits were generated using AlphaFold2⁶⁰ and AlphaFold-multimer⁶¹ through the ColabFold⁶² notebook. The calpain-2 heteromeric structure was annotated with the adduction sites identified with LC-MS/MS using UCSF ChimeraX.⁶³

MALDI-MS Sample Preparation and Analysis. Samples were separately desalted and concentrated with Amicon Ultra 3 kDa MWCO filters (Millipore-Sigma). The Amicon filters were prerinsed twice with LC-MS-grade water and centrifuged at 14,000g for 15 min at 4 °C. Separately, samples were added to the Amicon filters, centrifuged at 14,000g for 10 min, followed by three serial additions of LC-MS grade water, and concentrated to approximately 1 $\mu\text{g}/\mu\text{L}$. Each desalted sample (1 μL ; 1 μg of protein) was spotted directly on the target plate and air-dried. Then, 1 μL of a 90:10 mixture of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxy benzoic acid (SDHB) matrix (Bruker Daltonics, Billerica, MA) was overlaid on the spot and air-dried. 1 μL of Protein Calibrant II (Bruker Daltonics) was spotted, allowed to dry, and overlaid with 1 μL of the SDHB matrix. Analysis was performed on an UltrafleXtreme MALDI-TOF (Bruker Daltonics) mass spectrometer. Mass spectra were collected in the m/z range of 20,000–92,000 in positive ion linear mode using an ion source voltage of ~ 20 kV and a laser frequency of 2000 Hz.

The raw data were processed with FlexAnalysis software (version 3.4, Bruker Daltonics). External calibration was performed by using the aforementioned Protein Calibrant II on a spot adjacent to each sample, and peaks were detected by using the default centroid method settings in the FlexAnalysis software. Identified peak lists were exported from the FlexAnalysis software, and the identified peaks were annotated using R (v. 4.2.2) and RStudio with the *ggplot2* (v. 3.4.2) package. Raw spectra were converted and exported to *mzXML* format, and spectra were recreated and visualized utilizing the *readMzXMLData* (v. 2.8.2) and *ggplot2* packages in R ([Supporting Information](#)).

The number of lipid oxidation product modifications on calpain-2 catalytic and regulatory subunits was calculated using the following equation

$$y_{jk} = \text{average} \left(\frac{\text{measured}_{ijk} - \text{theoretical}_k}{\text{oxidant}_j} \right)$$

where y_{jk} is the average number of lipid oxidation product modifications of the i th replicate, j th oxidant, and k th calpain-2 subunit. measured_{ijk} is the observed mass (m/z) of the most intense peak associated with calpain-2. theoretical_k is the theoretical mass of calpain-2 catalytic (79 906 Da) and regulatory (28 068 Da) subunits from UniProt. oxidant_j is the mass of malondialdehyde (54.011 Da), 2-hexenal (80.063 Da), and 4-hydroxynonenal (154.115 Da).

Statistical Analysis. Calpain-2 activity was expressed as a percent of the control within each technical replicate ($n = 6$). The effect of each oxidant on the calpain-2 activity was determined separately. Calpain-2 activity data were analyzed using a one-way analysis of variance with R and RStudio, with the fixed effect of the oxidant concentration. Estimated marginal means were computed using the *emmeans* (v. 1.8.5) package to estimate the difference in the relative activity of calpain-2 due to the oxidant concentration. Statistical significance was denoted by a $P < 0.05$.

RESULTS AND DISCUSSION

Effect of Lipid Peroxidation Products on Calpain-2 Activity and Autolysis. Calpain-2 activity, measured using a caseinolytic assay, was differentially impacted by secondary lipid oxidation products ([Figure 1](#)). At the concentrations used

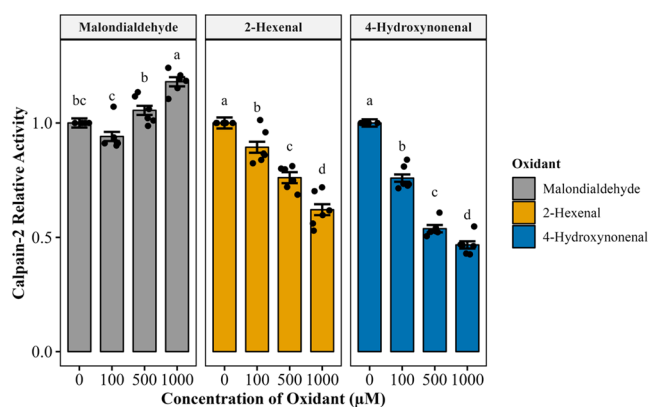


Figure 1. Purified porcine calpain-2 relative caseinolytic activity ($n = 6$) after 24 h incubation with 0 μM (buffer control) and 100, 500, or 1000 μM of (left to right) malondialdehyde, 2-hexenal, or 4-hydroxynonenal. Data are expressed as a percent of control within a technical replicate and are presented as estimated marginal means $\pm$ standard error. Means within an oxidant treatment without a common letter (a–d) are statistically different ($P < 0.05$).

in this study, malondialdehyde had a minimal impact on calpain-2 activity. A significant increase ($P < 0.05$) in calpain-2 activity compared to the control was observed only at the highest malondialdehyde concentration (1000 μM). Conversely, both 2-hexenal and 4-hydroxynonenal resulted in significantly lower ($P < 0.05$) calpain-2 activity versus the control at all oxidant incubation concentrations.

The impacts of lipid oxidation products on calpain-2 activity were similarly observed when assessing calpain-2 autolysis ([Figure 2](#)). Malondialdehyde ([Figure 2A](#)) incubation and subsequent calcium addition resulted in no visual differences in the disappearance of the calpain-2 catalytic (80 kDa) or regulatory (28 kDa) subunits or the appearance of an approximately 37 kDa autolysis product within each reaction time. Conversely, both 2-hexenal ([Figure 2B](#)) and 4-hydroxynonenal ([Figure 2C](#)) incubation and subsequent calcium addition resulted in less disappearance of the calpain-2 catalytic or regulatory subunits and less appearance of an approximately 37 kDa autolysis product within each reaction time point compared to the control incubations. The calpain-2 autolytic changes after CaCl_2 inclusion are similar to those observed previously, including the generation of the approximately 37 kDa autolysis product,^{64–66} and the lack of resolution of the unautolyzed (80 kDa) and autolyzed (78 kDa) calpain-2 catalytic subunits.⁶⁷

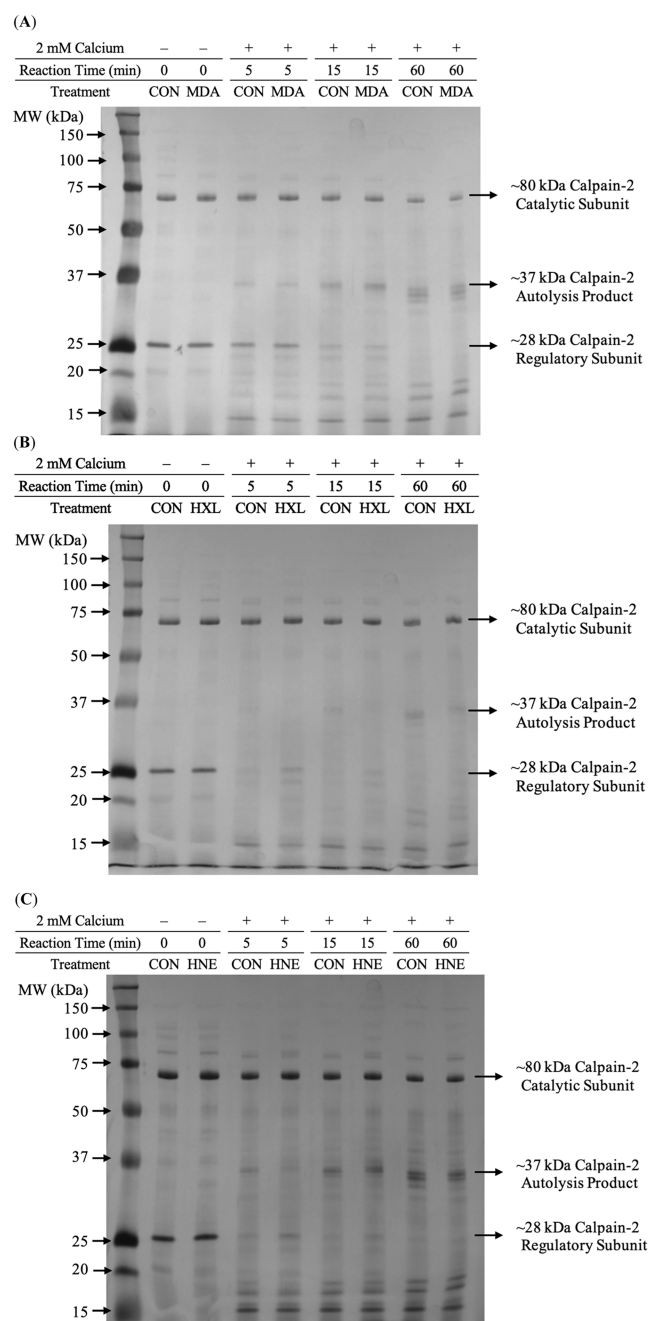


Figure 2. Representative silver-stained 15% sodium dodecyl sulfate-polyacrylamide gels of purified porcine calpain-2 after 24 h incubation with 1000 μ M of (A) malondialdehyde (MDA), (B) 2-hexenal (HXL), or (C) 4-hydroxynonenal (HNE) at 4 $^{\circ}$ C. Samples were removed from the reaction before adding CaCl_2 to serve as the no-calcium control (0 min) and at 5, 15, and 60 min after adding 2 mM CaCl_2 and immediately quenched in a protein-denaturing buffer. Each lane contains 1.5 μ g protein, and calpain-2 autolysis was evaluated within each reaction time point to assess differences in the disappearance of the 80 and 28 kDa subunits and the appearance of 37 kDa autolysis products between the control and lipid oxidation product incubated samples.

The differential impact of the secondary lipid oxidation products is noteworthy, as no oxidation product promoted enzyme activity by a relevant amount, nor did any oxidant completely impede or inhibit all calpain-2 functionality. This may suggest that 2-hexenal and 4-hydroxynonenal modify

residues near or adjacent to the active site but do not directly modify the residues within the active site. Similar results with calpain-1 were observed, where incubation with malondialdehyde at 100, 500, and 1000 μ M had minimal impact on calpain-1 activity and autolysis, while 2-hexenal and 4-hydroxynonenal decreased the activity at all concentration levels tested and slowed down calpain-1 autolysis.³²

Previous studies have shown that pyruvate kinase activity was reduced only by exposure to 5 mM malondialdehyde,¹¹ which is 5-fold greater than the greatest concentration used in this study. Similarly, lactate dehydrogenase and NADH-dependent metmyoglobin reductase activities were severely negatively impacted by separate incubations with 0.7 mM malondialdehyde, 2-hexenal, and 4-hydroxynonenal.⁶⁸ Additional studies with NADH-dependent metmyoglobin reductase have observed decreases in activity after incubation with 4-hydroxynonenal,²² while others have observed no differences with 2-hexenal or 4-hydroxynonenal.⁶⁹ Differences in experimental conditions, including the lipid oxidation product concentrations, reaction pH, and other incubation conditions, partly explain the differences in the effects of lipid oxidation products on enzyme activities within the aforementioned studies.

Adducts of Lipid Peroxidation Products on Calpain-2.

The reactivity and adduction of lipid oxidation products to proteins have been evaluated with mass spectrometry approaches over the last two decades. Specific oxidation product adduction sites on tryptic peptides have been identified *in vitro*, including 4-hydroxynonenal modifications on lactate dehydrogenase,¹⁰ cytochrome c,^{12,13} cytochrome c oxidase,¹⁴ hemoglobin,¹⁷ myoglobin,^{21,23,24,27} apomyoglobin,^{8,30,31} calpain-1,³² and soy protein isolate,⁷⁰ malondialdehyde modifications on pyruvate kinase,¹¹ calpain-1,³² and soy protein isolate,⁷⁰ and 2-hexenal modifications on model peptides.⁵ Many of these studies aimed to explain the mechanisms of the observed changes in protein function due to incubation with lipid oxidation products by identifying and quantifying the location and number of lipid oxidation products. In this study, we demonstrate that secondary lipid oxidation products impacted calpain-2 proteolytic activity and autolysis and thus aimed to elucidate the specific modifications that contribute to these observed impacts. Calpain-2 was incubated with the highest concentration of oxidant (1000 μ M) for the subsequent analysis by LC-MS/MS and MALDI-MS, as this concentration had the most significant impact on calpain-2 activity and autolysis, especially regarding 2-hexenal and 4-hydroxynonenal.

Previous studies have evaluated the mechanisms of chemical modifications of amino acid residues by lipid oxidation products, where the Michael adduction of 4-hydroxynonenal,^{32,71–73} Schiff base-type adducts of malondialdehyde,^{11,32,72} and Schiff base-type, pyridinium-type, and Michael adducts of 2-hexenal^{4–6} have been primarily observed. The current study observed Schiff base-type malondialdehyde and 2-hexenal modifications with LC-MS/MS. The adduction site, peptide sequence, precursor ion charge, and mass error are presented in Tables 1–3, and representative fragment spectra and ion tables are included in the Supporting Information. Following the incubation of calpain-2 with malondialdehyde, 36 and 10 distinct adduction sites on the catalytic and regulatory subunits, respectively, were identified. (Table 1). There were 8 and 1 distinct malondialdehyde adduction sites on the catalytic and regulatory subunits, respectively, identified in the

Table 1. Calpain-2 Residues Modified by Malondialdehyde Identified in Only the Treatment Samples^a

subunit and domain	modified residues	peptide position and sequence	treatment	
			Chrg. ^b	PPM ^c
Catalytic Subunit				
Domain IIa	Asn29	27-YLN ⁺⁵⁴ QDYAELRDQCLEAGALFQDPSFPALPSSLGFK ₆₁	+3	+3.53
Domain IIa	Gln30	27-YLNQ ⁺⁵⁴ DYAELRDQCLEAGALFQDPSFPALPSSLGFK ₆₁	+3	+3.01
Domain IIa	Gln30	27-YLNQ ⁺⁵⁴ DYAELRDQCLEAGALFQDPSFPALPSSLGFKELGPYSGK ₆₉	+4	+1.50
Domain IIa	Lys61	27-YLNQDYAELRDQCLEAGALFQDPSFPALPSSLGFK ₆₁ ⁺⁵⁴	+4	+2.43
Domain IIa	Lys61	37-DQCLEAGALFQDPSFPALPSSLGFK ₆₁ ⁺⁵⁴	+3	+3.85
Domain IIa	Lys61	37-DQCLEAGALFQDPSFPALPSSLGFK ⁺⁵⁴ ELGPYSGK ₆₉	+3	+2.31
Domain IIa	Gln129	124-VVPLDQ ⁺⁵⁴ SFQENYAGIFR ₁₄₀	+2	+4.13
Domain IIa	Lys183	162-DGELLFVHSAEGSEFWSALLEK ₁₈₃ ⁺⁵⁴	+3	+3.73
Domain IIa	Lys183	162-DGELLFVHSAEGSEFWSALLEK ⁺⁵⁴ AYAK ₁₈₇	+3	+3.72
Domain IIa	Asn189	188-IN ⁺⁵⁴ GCEALSGGATTEGFEDFTGGIAEWYELR ₂₁₈	+2	+4.41
Domain IIa	Asn189	188-IN ⁺⁵⁴ GCEALSGGATTEGFEDFTGGIAEWYELRK ₂₁₉	+3	+3.26
Domain IIa	Cys191	188-INGC ⁺⁵⁴ YEALSGGATTEGFEDFTGGIAEWYELR ₂₁₈	+3	+3.40
Domain IIa	Cys191	188-INGC ⁺⁵⁴ YEALSGGATTEGFEDFTGGIAEWYELRK ₂₁₉	+3	+2.23
Domain IIb	Gln233	231-ALQ ⁺⁵⁴ KGSLGCSIDITSAADSEAVTFQK ₂₅₇	+3	+3.11
Domain IIb	Lys234	231-ALQK ⁺⁵⁴ GSLGCSIDITSAADSEAVTFQK ₂₅₇	+3	+3.37
Domain IIb	Cys240	235-GSLLGC ⁺⁵⁴ SIDITSAADSEAVTFQK ₂₅₇	+2	+3.04
Domain IIb	Cys240	235-GSLLGC ⁺⁵⁴ SIDITSAADSEAVTFQKLVK ₂₆₀	+3	+3.25
Domain IIb	Gln256	235-GSLLGCSIDITSAADSEAVTFQ ⁺⁵⁴ KLVK ₂₆₀	+3	+3.57
Domain IIb	Lys257	235-GSLLGCSIDITSAADSEAVTFQK ₂₅₇ ⁺⁵⁴	+3	+4.57
Domain IIb	Lys257	235-GSLLGCSIDITSAADSEAVTFQK ⁺⁵⁴ LVK ₂₆₀	+3	+3.11
Domain IIb	Lys260	235-GSLLGCSIDITSAADSEAVTFQKLVK ₂₆₀ ⁺⁵⁴	+3	+3.62
Domain IIb	Lys260	258-LVK ⁺⁵⁴ GHAYSVTGAEVESR ₂₇₅	+2	+2.89
Domain IIb	Lys260	258-LVK ⁺⁵⁴ GHAYSVTGAEVESRGS ₂₈₀ LQK	+3	+4.05
Domain IIb	Gln279	261-GHAYSVTGAEVESRGS ₂₈₃ LQ ⁺⁵⁴ KLIR	+4	+4.65
Domain IIb	Lys280	261-GHAYSVTGAEVESRGS ₂₈₀ LQK ⁺⁵⁴	+3	+3.12
Domain IIb	Lys280	261-GHAYSVTGAEVESRGS ₂₈₃ LQK ⁺⁵⁴ LIR	+4	+4.14
Domain IIb	Arg285	284-IR ⁺⁵⁴ NPWGEVEWTGQW ₃₁₂ NDNC ₃₁₂ PNWNTVDPEVR	+3	+2.89
Domain IIb	Arg318	318-R ⁺⁵⁴ HEDGEFWMSFSD ₃₃₃ FLR	+3	+4.13
Domain IIb	Asn342	338-LEICN ⁺⁵⁴ LTPDTLTSDSYK ₃₅₅ K	+3	+4.50
Domain IIb	Lys354	338-LEICNLTPDTLTSDSYK ⁺⁵⁴ K ₃₅₅	+2	+4.37
Domain IIb	Lys355	338-LEICNLTPDTLTSDSYK ₃₅₅ ⁺⁵⁴	+2	+4.08
Domain III	Asn376	376-N ⁺⁵⁴ YPNTFWMNPQY ₃₉₀ LIK	+2	+4.47
Domain III	Gln398	391-LEEDEDQ ⁺⁵⁴ EDGESGCTFLVGLIQK ₄₁₄	+2	+3.27
Domain III	Gln398	391-LEEDEDQ ⁺⁵⁴ EDGESGCTFLVGLIQKHR ₄₁₆	+4	+3.27
Domain III	Lys414	391-LEEDEDQEDGESGCTFLVGLIQK ⁺⁵⁴ HR ₄₁₆	+3	+0.72
Domain III	His415	391-LEEDEDQEDGESGCTFLVGLIQKH ⁺⁵⁴ R ₄₁₆	+4	+3.56
Domain III	Lys421	421-K ⁺⁵⁴ MGEDMHTIGFGIYVPEELTGQTNIHLSK ₄₅₀	+3	+2.81
Domain III	His427	421-KMGEDMH ⁺⁵⁴ TIGFGIYVPEELTGQTNIHLSK ₄₅₀	+4	+2.89
Domain III	His427	422-MGEDMH ⁺⁵⁴ TIGFGIYVPEELTGQTNIHLSK ₄₅₀	+2	+1.76
Domain III	Gln443	421-KMGEDMHTIGFGIYVPEELTGQ ⁺⁵⁴ TNIHLSK ₄₅₀	+4	+3.38
Domain III	His447	421-KMGEDMHTIGFGIYVPEELTGQTNIH ⁺⁵⁴ LSK ₄₅₀	+4	+3.09
Domain III	Lys450	421-KMGEDMHTIGFGIYVPEELTGQTNIHLSK ₄₅₀ ⁺⁵⁴	+4	+2.97
Domain III	Lys450	422-MGEDMHTIGFGIYVPEELTGQTNIHLSK ₄₅₀ ⁺⁵⁴	+4	+3.64
Domain III	Asn473	470-EVLN ⁺⁵⁴ RFKLPPGEYILVPSTFEPNK ₄₉₃	+3	+3.63
Domain III	Arg474	470-EVLNR ⁺⁵⁴ FKLPPGEYILVPSTFEPNK ₄₉₃	+3	+3.93
Domain III	Lys506	506-K ⁺⁵⁴ ADYQVVDDEIEADLEENDASEDDIDDGFRR ₅₃₆	+3	+0.67
Domain III	Gln510	506-KADYQ ⁺⁵⁴ VVDDEIEADLEENDASEDDIDDGFRR ₅₃₆	+4	+3.23
Domain IV	Lys578	569-SDGFSIETCK ⁺⁵⁴ IMVMDLSDSGSGK ₅₉₁	+3	+4.00
Domain IV	Arg618	613-EIDVDR ⁺⁵⁴ SGTMNSYEMRK ₆₂₉	+3	+4.80
Domain IV	Lys629	613-EIDVDRSGTMNSYEMRK ₆₂₉ ⁺⁵⁴	+3	+4.73
Regulatory Subunit				
Domain V	Asn27	10-GGGGGGGGGGGLGGGLGN ⁺⁵⁴ VLGGLISAGGGGGGGGGGGGGGGGGTAMR ₅₈	+3	+2.01
Domain V	His84	83-TH ⁺⁵⁴ YSNIEANESEEV ₉₇ R	+2	+4.30
Domain V	Asn87	83-THYSN ⁺⁵⁴ IEANESEEV ₉₇ R	+3	+4.92
Domain V	Arg101	101-R ⁺⁵⁴ LFAQLAGDDMEVSATELMNILNK ₁₂₄	+2	+3.48

Table 1. continued

subunit and domain	modified residues	peptide position and sequence	treatment	
			Chrg. ^b	PPM ^c
Catalytic Subunit				
Domain VI	Gln105	¹⁰¹ RLFAQ ⁺⁵⁴ LAGDDMEVSATELMNILNK ₁₂₄	+3	+3.30
Domain VI	Gln105	¹⁰² LFAQ ⁺⁵⁴ LAGDDMEVSATELMNILNK ₁₂₄	+2	+3.65
Domain VI	Arg128	¹²⁵ VVTR ⁺⁵⁴ HPDLKTDGFGIDTCR ₁₄₃	+3	+4.44
Domain VI	Cys142	¹³⁴ TDGFGIDTC ⁺⁵⁴ RSMVAVMDSDTTGKLGFEFEK ₁₆₃	+3	+2.79
Domain VI	Lys156	¹⁴⁴ SMVAVMDSDTTGK ⁺⁵⁴ LGFEFEK ₁₆₃	+2	+4.40
Domain VI	Arg214	²¹⁴ R ⁺⁵⁴ YSDEGGNMDFDNFISCLVR ₂₃₃	+3	+4.55
Domain VI	Asn221	²¹⁵ YSDEGGN ⁺⁵⁴ MDFDNFISCLVR ₂₃₃	+2	+3.51

^aThe treatment samples were incubated for 24 h with 1 mM malondialdehyde. ^bChrg. = precursor ion charge. ^cPPM = precursor mass error expressed as parts per million.

Table 2. Calpain-2 Residues Modified by Malondialdehyde Identified in the Control and Treatment Samples or Only in the Control Samples^a

subunit and domain	modified residues	peptide position and sequence	control		treatment	
			Chrg. ^b	PPM ^c	Chrg. ^b	PPM ^c
Catalytic Subunit						
Domain IIa	Gln38 Gln47	³⁷ DQ ⁺⁵⁴ CLEAGALFQ ⁺⁵⁴ DPSFPALPSSLGFK ₆₁	+3	+1.24		
Domain IIa	Gln38	³⁷ DQ ⁺⁵⁴ CLEAGALFQDPSFPALPSSLGFK ₆₁			+3	+3.43
Domain IIa	Cys39	³⁷ DQC ⁺⁵⁴ LEAGALFQDPSFPALPSSLGFK ₆₁	+3	+4.07	+2	+3.57
Domain IIa	His169	¹⁶² DGELLFVH ⁺⁵⁴ SAEGSEFWSALLEK ₁₈₃	+3	+4.38	+2	+4.46
Domain IIb	His262	²⁵⁸ LVKGH ⁺⁵⁴ AYSVTGAEVESR ₂₇₅	+3	+0.96	+3	+4.63
Domain IIb	His262	²⁵⁸ LVKGH ⁺⁵⁴ AYSVTGAEVESRGS ₂₈₀ LQK ₂₈₀			+4	+3.87
Domain IIb	His262	²⁶¹ GH ⁺⁵⁴ AYSVTGAEVESRGS ₂₈₀ LQK ₂₈₀			+3	+4.63
Domain IIb	Asn286	²⁸⁴ IRN ⁺⁵⁴ PWGEVEWTGQWNDNCPNWNNTVDPEVR ₃₁₂	+3	+3.20	+3	−1.87
Domain IIb	Asn286	²⁸⁶ N ⁺⁵⁴ PWGEVEWTGQWNDNCPNWNNTVDPEVR ₃₁₂			+3	+1.22
Domain IIb	Cys301	²⁸⁶ NPWGEVEWTGQWNDNC ⁺⁵⁴ PNWNNTVDPEVR ₃₁₂	+3	+1.38		
Domain IIb	His319	³¹⁸ RH ⁺⁵⁴ EDGEFWMSFSDFLR ₃₃₃	+3	+4.49	+2	+4.03
Domain IIb	His319	³¹⁹ H ⁺⁵⁴ EDGEFWMSFSDFLR ₃₃₃			+2	+4.29
Domain IIb	Cys341	³³⁸ LEIC ⁺⁵⁴ NLTPD ₃₅₄ TLTSDSYK ₃₅₄			+2	+4.59
Domain IIb	Cys341	³³⁸ LEIC ⁺⁵⁴ NLTPD ₃₅₅ TLTSDSYKK ₃₅₅	+3	−3.60	+2	+4.01
Domain III	Lys476	⁴⁷⁵ FK ⁺⁵⁴ LPPGEYILVPSTFEPNK ₄₉₃	+2	+4.71	+2	+4.27
Domain III	Lys476	⁴⁷⁰ EVLNRFK ⁺⁵⁴ LPPGEYILVPSTFEPNK ₄₉₃			+3	+4.13
Regulatory Subunit						
Domain V	Gln73	⁵⁹ ILGGVISIAISEAAAQ ⁺⁵⁴ YNPEPPPPR ₈₂	+3	+4.69	+3	+4.53

^aThe treatment samples were incubated for 24 h with 1 mM malondialdehyde. ^bChrg. = precursor ion charge. ^cPPM = precursor mass error expressed as parts per million.

Table 3. Calpain-2 Residues Modified by 2-Hexenal Identified in Either the Control and Treatment Samples or Only in the Treatment Samples^a

subunit and domain	modified residues	peptide position and sequence	control		treatment	
			Chrg. ^b	PPM ^c	Chrg. ^b	PPM ^c
Catalytic Subunit						
Domain IIa	Cys39	³⁷ DQC ⁺⁸⁰ LEAGALFQDPSFPALPSSLGFK ₆₁	+2	+0.68	+2	+0.62
Domain IIb	Asn298	²⁸⁴ IRNPWGEVEWTGQWN ⁺⁸⁰ DNCPNWN ₃₁₂			+3	−0.35
Domain IIb	Asn300	²⁸⁴ IRNPWGEVEWTGQW _{NDN} ⁺⁸⁰ CPNWN ₃₁₂			+4	+1.74
Domain IIb	Cys301	²⁸⁶ NPWGEVEWTGQW _{NDNC} ⁺⁸⁰ PNWN ₃₁₂			+3	+1.61

^aThe treatment samples were incubated for 24 h with 1 mM 2-hexenal. ^bChrg. = precursor ion charge. ^cPPM = precursor mass error expressed as parts per million.

control and malondialdehyde-incubated samples (Table 2). Additionally, 2 distinct malondialdehyde adduction sites were identified in only the control samples (Table 2). After the incubation of calpain-2 with 2-hexenal, 3 distinct adduction sites on the catalytic subunit were identified only in the 2-hexenal incubated samples, and 1 distinct adduction site was

identified in both the control and 2-hexenal incubated samples (Table 3). No specific adduction sites of calpain-2 were identified following incubation with 4-hydroxynonenal.

The identified adduction site residues were annotated on the AlphaFold2^{60,61} predicted porcine calpain-2 heteromeric structure, and the structure colored by the pLDDT model

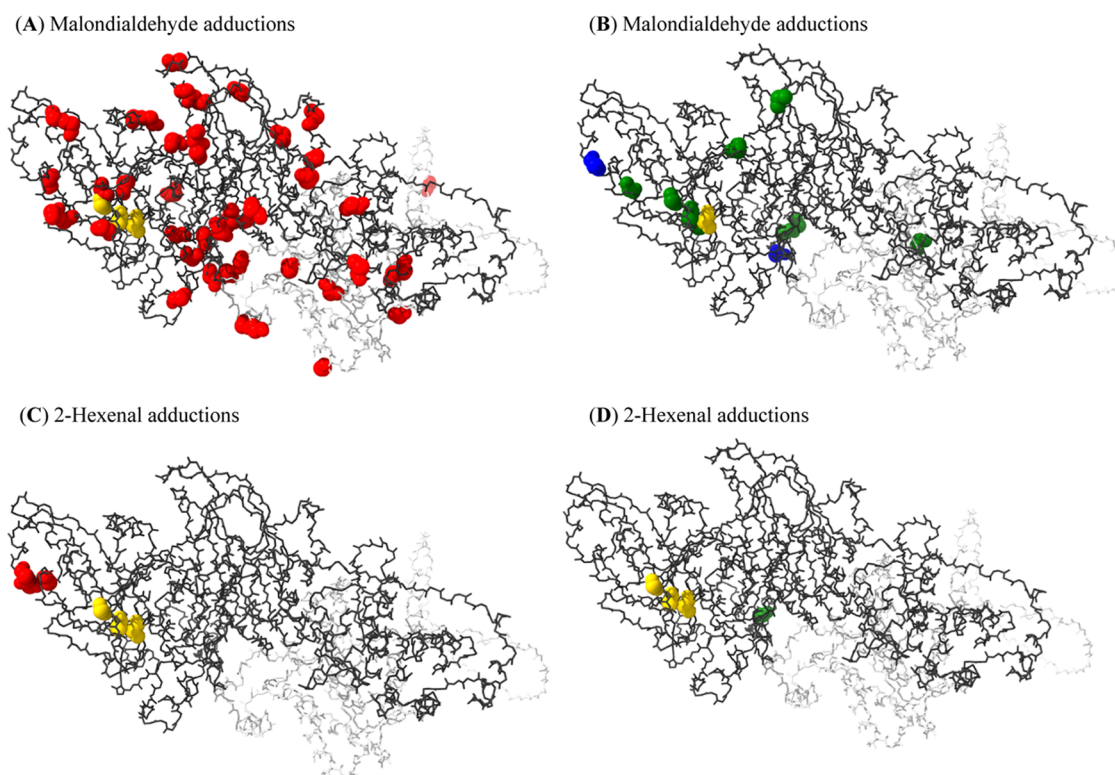


Figure 3. Calpain-2 structure with annotations of modifications. The peptide backbone is presented with the catalytic and regulatory subunits colored in gray and white, respectively. The residues modified by the lipid oxidation products as identified by LC-MS/MS are presented in the space-fill form, with the active site residues colored gold. (A) Malondialdehyde and (C) 2-hexenal adductions (red) were identified only in the lipid oxidation incubated samples. (B) Malondialdehyde and (D) 2-hexenal adductions were identified in both the control and incubated samples (green) and only in the control (blue). To view the colored version, please refer to the online journal article.

confidence scores is presented (Supporting Information Figure 1). The adducted residues are displayed in the space-fill and colored form according to the identification in either the treatment or control groups for malondialdehyde (Figure 3A,B) and 2-hexenal (Figure 3C,D). The adducted residues are located almost exclusively on the exterior surface of the predicted calpain-2 structure. Many of the malondialdehyde modifications were identified only in the calpain-2 samples incubated with malondialdehyde (Table 1), while some adduction sites were identified in the control and malondialdehyde-incubated samples (Table 2). Previous work with calpain-1 also identified some adduction sites shared between the control and lipid oxidation product incubated samples.³² While the initial muscle tissue was collected immediately post-mortem, homogenized, clarified, and subjected to dialysis, some lipid oxidation products may already be present in the muscle tissue that could bind to calpain-2.

Quantification of the Number of Secondary Lipid Binding Sites. Identifying specific adduction sites with LC-MS/MS allows for a greater understanding of the mechanisms by which lipid oxidation products impact protein function. While LC-MS/MS-based approaches identify specific adduction sites on a protein, they do not discriminate the number of modifications on the protein population. Intact protein analysis by MALDI-MS, however, enables the quantification of the number of modifications within the protein population. This approach has been successfully used to quantify the number of 4-hydroxynonenal modifications on lactate dehydrogenase,¹⁰ cytochrome c,^{12,13} cytochrome c oxidase,¹⁴ β -lactoglobulin B,^{15,16} hemoglobin,^{15,17} insulin,^{3,19} myoglobin,^{20–24,27,29} and

apomyoglobin,^{8,30} and the number of 2-hexenal modifications on cytochrome c.^{4,6} LC-MS/MS and MALDI-MS offer distinct, yet complementary, data to evaluate specific and quantitative changes related to these and other protein modifications.

The current study used MALDI-MS to quantify (Figure 4A) the intact masses of the calpain-2 catalytic (Figure 4B) and regulatory (Figure 4C) subunits. Data were collected in positive linear ionization mode; thus, the diversity in lipid oxidation product modifications on calpain-2 could not be resolved (Supporting Information Figures 2–5). Interestingly, no increases in the intact protein mass were observed when calpain-2 was dialyzed against a reducing buffer with 2-mercaptoethanol before incubation with secondary lipid oxidation products. Conversely, when calpain-2 was dialyzed against a buffer without a reducing agent, the intact protein masses increased from the expected unmodified calpain-2 subunit masses.

Approximately 25–32 and 8–10 malondialdehyde modifications on the calpain-2 catalytic and regulatory subunits, respectively, were observed by MALDI-MS (Figure 4). Previous *in vitro* studies on lactate dehydrogenase (37 kDa),¹⁰ myoglobin (18 kDa),^{21,24,27} β -lactoglobulin B (18 kDa),¹⁵ hemoglobin α chain (15 kDa),¹⁵ cytochrome c (12 kDa),¹³ and insulin (12 kDa)¹⁹ have observed about 1–8 HNE adducts. Extrapolating on a molecular weight basis, the observation of 25–32 and 8–10 malondialdehyde modifications on calpain-2 catalytic and regulatory subunits is generally consistent with the previously published works.^{10,15} The MALDI-MS results are in strong agreement with the LC-

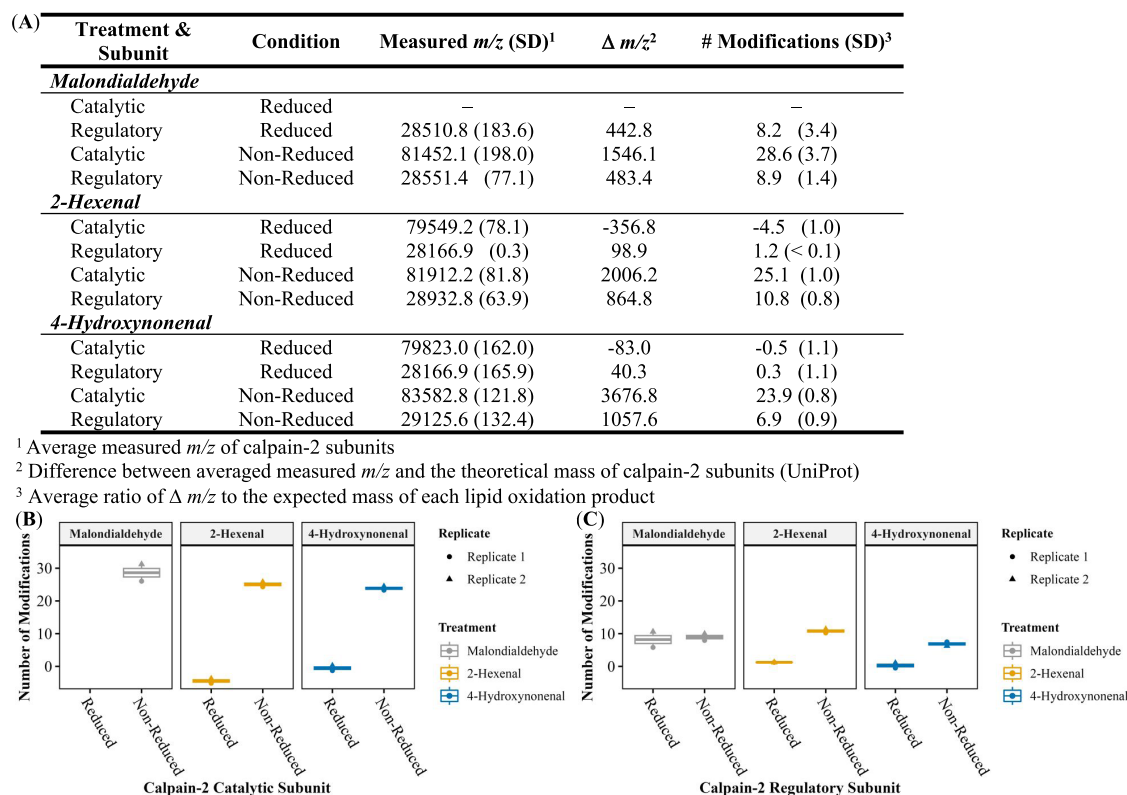


Figure 4. (A) Quantification of the number of lipid oxidation product modifications on calpain-2 catalytic and regulatory subunits was performed using the equation $y_{jk} = \text{average} \left(\frac{\text{measured}_{ijk} - \text{theoretical}_k}{\text{oxidant}_j} \right)$, where y_{jk} is the average number of lipid oxidation product modifications of the i th replicate, j th oxidant, and k th calpain-2 subunit. Graphical representation of the number of lipid oxidation product modifications on calpain-2 (B) catalytic and (C) regulatory subunits based on the dialysis of calpain-2 in the presence (reduced) or absence (nonreduced) of 2-mercaptoethanol before incubation with lipid oxidation products.

MS/MS identification of malondialdehyde-modified peptides, where 36 and 10 distinct modifications on malondialdehyde-incubated calpain-2 catalytic and regulatory subunits, respectively, were observed. Various species of malondialdehyde-modified calpain-2 are likely present in the protein population as adjacent residues (e.g., Lys354 and Lys355) may be codependent. A mechanistic evaluation of the progression of lipoxidation on calpain-2 is outside the scope of the current study, but such experiments could identify or better explain specific residues crucial for normal protein function against those modified residues with minimal impact on normal protein function.

Consistent observation of 2-hexenal and 4-hydroxynonenal modification of calpain-2 was dependent on dialysis against a buffer without a reducing agent. Approximately 24–25 2-hexenal and 23–25 4-hydroxynonenal modifications on the calpain-2 catalytic subunit (Figure 4A,B), and approximately 10–11 2-hexenal and 6–8 4-hydroxynonenal modifications, were observed on the calpain-2 regulatory subunit (Figure 4A,C). These observations are generally consistent with previous works utilizing lower-molecular-weight proteins.^{10,15} Despite limited success in identifying specific adduction sites of 2-hexenal and 4-hydroxynonenal by LC-MS/MS, these data demonstrate that significant modifications of calpain-2 occur. Some modified residues were identified in the control and lipid oxidation incubated samples (Tables 2 and 3). While these modifications were observed, a substantial number of modifications are unlikely to exist on individual protein

molecules as minimal shifts in the intact mass of control samples from the expected mass were observed (Supporting Information, Figure 2).

The estimated number of modifications on calpain-2 by 2-hexenal and 4-hydroxynonenal based on the MALDI-MS results is not consistent with the LC-MS/MS results. An explanation for the inconsistency may be partly attributed to incomplete or impeded trypsin digestion, shifts in expected retention times, the lack of ionization efficiency, or the resulting neutral loss and modification instability. As trypsin cleaves arginine and lysine residues and 2-hexenal and 4-hydroxynonenal bind to these residues, the modification may prevent or preclude the accessibility of trypsin for proteolytic cleavage. Previous studies described above have achieved success with trypsin digestion in localizing 4-hydroxynonenal modifications, while other studies have utilized alternative enzymes alone or in combination with trypsin, such as chymotrypsin^{8,12,13} and trypsin/Asp-N.¹⁰ Alternative enzyme digestions could generate peptides more suitable for analysis by LC-MS/MS. Other fragmentation losses are likely to occur as neutral losses, and alternative fragmentation strategies, such as electron transfer dissociation, can potentially retain some labile modifications, including 4-hydroxynonenal.⁷⁴ The utility of these approaches may be experimental-, protein-, and peptide-dependent, but future work should consider these when designing, conducting, and analyzing experimental data.

These data demonstrate the differential impact of specific lipid oxidation products on calpain-2 activity and the

progression of autolysis *in vitro*. LC-MS/MS identified specific adduction site modifications, including many malondialdehyde and some 2-hexenal modifications. Intact protein mass analysis validated that calpain-2 catalytic and regulatory subunits become heavily modified due to secondary lipid oxidation products. The degree to which calpain-2 becomes modified in living and post-mortem skeletal muscle remains unknown. The susceptibility of calpain-2 to modification by lipid oxidation products emphasizes that these modifications could alter critical cellular events, such as myoblast fusion, apoptotic pathways, and other signaling pathways. The disruption of these cellular events by lipid oxidation products may profoundly impact normal cell function and survival and be another important source of variation to consider in subsequent experiments. Lipid oxidation product modifications may also impact calpain-2 in a post-mortem skeletal muscle system, as calpain-2, compared with calpain-1, is active during extended post-mortem aging periods. Lipid oxidation products generally accumulate during extended post-mortem aging and could interact with proteins over prolonged exposure to the cellular environment. Thus, it could be hypothesized that these lipid oxidation products have the potential to modify and impact calpain-2 *in vivo*. If calpain-2 is modified to the degree calculated with MALDI-MS, these modifications may impact the localization or solubility of calpain-2 by altering the chemical properties of the protein. However, subsequent research is needed to evaluate the extent and frequency of these modifications in various cellular environments. Overall, these *in vitro* results add to the understanding of modifications impacting calpain-2 and provide another source of variation to be considered in subsequent experiments.

■ ASSOCIATED CONTENT

Data Availability Statement

The raw LC-MS/MS and MALDI data files and the Proteome Discoverer output files were deposited in the ProteomeX-change consortium via the MassIVE⁷⁵ partner repository with the identifier PDX045905.

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.4c00335>.

Representative spectra of modified peptides with fragment ion tables (PDF)

The projected *Sus scrofa* calpain-2 heteromeric structure through AlphaFold2 and Multimer and MALDI-MS spectra (PDF)

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Notes

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